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Induction of *Rhizopus oryzae* Pyruvate Decarboxylase Genes

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Abstract. Two pyruvate decarboxylase genes, *pdcA*, and *pdcB*, were cloned from *Rhizopus oryzae*. These genes are similar to each other with approximately 85% nucleotide sequence identity within the coding region. Multiple transcriptional start sites and polyadenylation sites were found for both genes. The deduced translation product of each gene results in a 561 amino acid protein with approximate molecular weight of 61 kDa each. The amino acid identity between the two proteins was 91% as calculated by Lipmann-Pearson comparisons. Transcriptional control appears to be important in regulation of the PDC, since much of the transcript accumulation parallels enzymatic activity. There was no detectable *pdc* transcript from cultures grown in glycerol-containing medium. Induction of transcription for *pdcA* and *pdcB* was initiated within 1.5 h of adding glucose to the culture. Shifting the aerobically grown cultures to anoxic conditions at this time resulted in enhanced *pdc* transcription, PDC enzymatic activity, and ethanol production, compared to cultures with continued aerobic growth.

The filamentous fungus *Rhizopus* is an obligate aerobe that is often used for industrial production of L-(+)-lactic acid, which currently has an estimated global market in excess of 100,000 tons per year [10]. Approximately 75% of the lactic acid is used in the food industry as an acidulant for flavor or as an antimicrobial agent. It is anticipated that demand for this organic acid will grow substantially with the introduction of the nonchlorinated solvent ethyl lactate and the biodegradable plastic polylactic acid [4, 11]. Some advantages of using *Rhizopus* species as an alternative to lactic acid bacteria include production of optically pure lactic acid, which is preferred for many applications, and the ability of the fungus to grow in a chemically defined minimal medium without the need for complex components such as yeast extract [27]. These supplements add significant cost and complicate purification of the final product. However, the shortcoming of using *Rhizopus* is that the production efficiency is still generally considered low compared to bacterial fermentations.

*Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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In an effort to better understand the fermentation control mechanisms of *Rhizopus*, I recently cloned an NAD^+-dependent lactate dehydrogenase (LDH; EC1.1.1.27) that appears to be primarily responsible for the conversion of pyruvate to lactic acid [28]. Under aerobic conditions of growth, more than 75% of the carbon is diverted through this pathway for synthesis of lactic acid [20, 30]. However, much of this metabolic flux is shunted into ethanol production under anaerobically stressed growth conditions that may result from inadequate aeration or mycelial clumping [29]. Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyzes the conversion of pyruvate to acetaldehyde, which is subsequently reduced to ethanol by alcohol dehydrogenase (ADH, EC 1.1.1.1). Zygomycetes fungi (e.g., *Rhizopus* and *Mucor*) are well known for their ability to ferment glucose to ethanol efficiently, but most research to date has focused primarily on the isolation and study of the ADH enzyme [9, 34]. The objectives of this study were to better elucidate the first conversion step of pyruvate to ethanol during anoxic growth. I have cloned and studied the expression of two pyruvate decarboxylase genes, *pdcA* and *pdcB*, from *R. oryzae* and have begun to relate their regulation to the production of ethanol and lactic acid.

Materials and Methods

Isolation of *pdc* genes. *R. oryzae* (syn. *R. arrhizus*) NRRL 395 was grown at 30°C in synthetic *Rhizopus* "Medium D" described by Treen-Sears et al. [32] to promote expression of ethanolic genes. This medium, containing 2 g glucose/L, was chosen because it promotes ethanol fermentation and only trace amounts of lactic acid are produced (data not shown). After 16 h growth, an additional 25 g glucose/L was added and the medium volume was adjusted to minimize air space within the flask. The flask was covered with a rubber septum vented with a 22 G needle to exhaust carbon dioxide and incubation continued for an additional 6 h. RNA isolated by a hot phenol method [3] from the anaerobically stressed culture served as template for cDNA synthesis using the SuperScript Preamplification System for First Strand Synthesis (Life Technologies, Gaithersburg, MD).

Degenerate nucleotide primers were designed to anneal to regions encoding conserved amino acid sequences found in several fungal PDC proteins [6, 12–14, 18, 26]. These regions correspond with thiamine diphosphate binding motifs that are localized around amino acids Asp-28 and Asp-444 for *S. cerevisiae* Pdc1 [8]. AmpliTaq (Applied Biosystems, Foster City, CA) PCR amplification with primers PDC1 5′-TTY GGT KTT CCT GGT GAY TWY AAC-3′ and PDC3 5′-TTC AAT WGT RTA ACC RYC RTT RTT-3′ were performed with the first strand cDNA as template. Conditions were as described by Ausubel et al. [3], except that the following program was used: 30 cycles of 95°C for 45 s, 55°C for 60 s, and 72°C for 90 s. The predicted 1.3-kb fragment was recovered and cloned into pCRII/TOPO (Invitrogen, Carlsbad, CA). Sequence analysis confirmed that the fragment represented a partial *pdc* gene. A probe was prepared from this fragment and used to isolate hybridizing clones from genomic and cDNA libraries described previously [28]. Southern analyses used the Genius system (Boehringer Mannheim, Indianapolis, IN) as recommended by the manufacturer for random primed labeling with digoxigenin.

Multiple protein sequence comparisons were performed as Clustal analyses using Lasergene Megalign (DNA Star, Madison, WI). The aligned sequences were further arranged by neighbor joining with bootstrap analysis and tree construction using TreeCon [33]. Default program settings were used for all analyses.

Induction of *pdc* genes. *R. oryzae* was grown from spores for 24 h with shaking at 30°C in a pre-induction RZ medium [28] containing 15 g glycerol/L and 5 g trypticase peptone/L (Becton Dickinson, Cockeysville, MD). It was previously shown that this medium does not support synthesis of detectable levels of ethanol or lactate. The culture was divided into six 1-L bubble column fermentors, equipped with automatic pH control, and glucose was added to a final concentration of 25 g/L in a total of 800 ml. Fermentors were initially aerated by sparging with compressed air through a fritted dispersion tube at a rate of 1 L/min. Dissolved oxygen was measured as percent saturation using pO_2 electrode (Ingold, Columbus, OH). Probes were calibrated with uninoculated medium sparged with air for the 100% saturation and sparged with nitrogen (purity >99.99%) for the 0% saturation. The pH was maintained at 5.5 by the addition of NaOH. After 1.5 h following the addition of glucose, one set of the fermentors was shifted from air to nitrogen in order to test the effects of anaerobic stress. Samples were taken every 1.5 h for the analysis of fermentation products and the isolation of protein and RNA. Mycelium from the overnight glycerol grown culture represented the non-induced control.

Glycerol and glucose concentrations in the medium were analyzed by HPLC using an HPX-87H column (Bio-Rad Laboratories) and a Waters 410 Differential Refractometer (Milford, MA). Ethanol measurements were performed in triplicate using the Sigma (St. Louis, MO) enzymatic alcohol detection kit (Product No. 333-UV).

Analyses of RNA. Northern analysis was performed with 3′-end digoxigenin labeled oligonucleotide probes that were designed to preferentially hybridize to either the *pdcA* or *pdcB* transcript. Primers PDCA 5′-GGT GAC GGA TGC GAT CGC CAA AGC CCA ATT C-3′ and PDCB 5′-CCG CTG TCA GAA ACC CGT CCA AGA GTT CAT CAC C-3′ each have at least a 44% mismatch with the target sequence of the opposite *pdc* gene. The oligonucleotide probes were analyzed to rule out cross annealing by performing hybridizations against increasing amounts of anti-sense PDCA and PDCB primers bound to membranes. The quality of the RNA and probe specificity were tested on individual denaturing formaldehyde gels containing 10 μg of RNA per sample and were analyzed separately with each probe. A 28S rRNA fragment from the same *Rhizopus* strain served as an internal standard to avoid quantitation anomalies associated with hypoxic and metabolic shifts in the culture [31, 35]. Hybridizations were performed in Ultrahyb Buffer (Ambion, Austin, TX) at 42°C, while washes were performed with a final stringency of 0.5 × SSC at 45°C [3]. The net intensities of hybridization signals obtained by northern analyses were determined using the Kodak 1D Image Analysis Software (Eastman-Kodak, Rochester, NY). The sum net intensities for each set of Northern analyses were normalized to an arbitrary value of 100%, so that each hybridization signal from a RNA sample represents a relative amount of total signal throughout the 6 h experiment. Values for each RNA sample from the same time point were then averaged.

Identification of the 5′ and 3′ untranslated regions of the *pdc* genes was performed using a GeneRacer Kit (Invitrogen, Carlsbad, CA) for random amplification of cDNA ends. The GeneRacer kit was used according to the manufacturer's recommendations for identification of the transcriptional start sites and polyadenylation regions. RNA isolated from the glucose-induced culture served as template and oligo dT primers were used to reverse transcribe the ligated mRNA using AMV-RT. Amplification of full length cDNA ends was performed using the GeneRacer 5′ and 3′ primers in combination with primers in the *pdc* genes. Amplified fragments were cloned and approximately 15–20 isolates from each amplification were sequenced.

Enzymatic analyses. Mycelial protein extracts were prepared in PDC extraction buffer (0.1 M 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.2, 10 ml/L glycerol, 1 mM dithiothreitol, 1 mM thiamine diphosphate, 2 mM $MgCl_2$) using a FastPrep System (Bio101, Vista, CA) with 0.5-mm zirconia-silica beads and then centrifuged at 15,800*g*, 15 min, at 4°C. PDC activity was assayed at 32°C by measuring the first order change in absorbance at 340 nm resulting from the oxidation of NADH. Reactions were performed in PDC assay buffer (0.1 M MES pH 6.2, 175 μM NADH, 2 mM $MgCl_2$, 1 mM thiamine diphosphate, 0.04 Units yeast ADH/μl (Sigma, St. Louis, MO), 25 mM potassium oxamate to inhibit LDH activity) and were initiated by the addition of sodium pyruvate to a final concentration of 4 mM. All protein concentrations were adjusted to ensure that the change in absorbance followed first order kinetics for a minimum of 3–5 min. Assays were performed in triplicate and one unit of enzyme activity is defined as the amount activity necessary to convert 1 μmole NADH to NAD^+ per min. Protein concentrations were determined using the BioRad Protein Assay kit (Hercules, CA).

Results

Isolation of *pdc* genes. I concluded that the 1.3-kb PCR fragment made with degenerate primers represented a partial *pdc* gene, since BlastX E-value scores [1] were approximately 10^{-80}. Screening the genomic library using this fragment as probe resulted in the isolation of two

closely related *pdc* genes, *pdcA* (GenBank Accession number AF282846) and *pdcB* (AF282847). These genes are similar to each other with approximately 85% nucleotide sequence identity within the coding region. Only *pdcA* was detected in the cDNA library. Sequence comparison with the genomic isolate revealed that there is a 52-bp intron 90 bp downstream of the proposed start codon. Sequence from the RACE analyses showed that a similarly located 55-bp intron is also present in *pdcB.* The sequence of the 1.3-kb probe was identical to the *pdcA* gene, although cross-hybridization did occur with *pdcB* due to the high nucleotide sequence identity with this long probe. Hybridization of total genomic DNA to the 1.3-kb *pdcA* probe resulted in detection of only *pdcA* and *pdcB* when using restriction enzymes, such as *Bam*HI and *Hind*III that do not cut within either gene (data not shown).

Sequence of the amplified products from the RACE analyses showed that both genes had multiple transcriptional start sites and polyadenylation sites. The *pdcA* had two initiation sites in close proximity at −37 and −34 upstream of the start codon and the *pdcB* had initiation sites at −53, −44, and −27. Polyadenylation sites were located +81, +86, and +94 downstream of the *pdcA* stop codon. Polyadenylation sites for the *pdcB* were located +95 and +100 downstream of the stop codon. There did not appear to be any noticeable correlation between the locations of transcript start sites and oxygen availability, or the time from glucose induction (data not shown).

The deduced translation product of each gene results in a 561 amino acid protein with an approximate molecular weight of 61 kDa. The amino acid identities between the two proteins were 91% as calculated by Lipmann-Pearson comparisons. These were also compared with the sequences of other PDC subunits (Fig. 1) and found to have similarities ranging from 29% for the *Zymomonas mobilis pdc* to approximately 40% for *Aspergillus nidulans*, *A. oryzae*, and all of the yeast PDC. The fungal and bacterial PDC are clustered together in a very unusual manner with *Aspergillus* spp. separated and *Neurospora crassa* having more similarity to *Z. mobilis* than the fungi.

Induction of *pdc* genes. Growth in the pre-induction medium was such that prior to the glucose induction, there was approximately 2 g glycerol/L remaining in the culture broth. The oxygen demand for the culture, grown in this non-fermentable substrate, was high and dissolved oxygen levels were approximately at 5–8% saturation prior to adding glucose. These levels did not significantly change during the first 1.5 h after the addition of glucose, when all of the cultures were aerated. Cultures that were

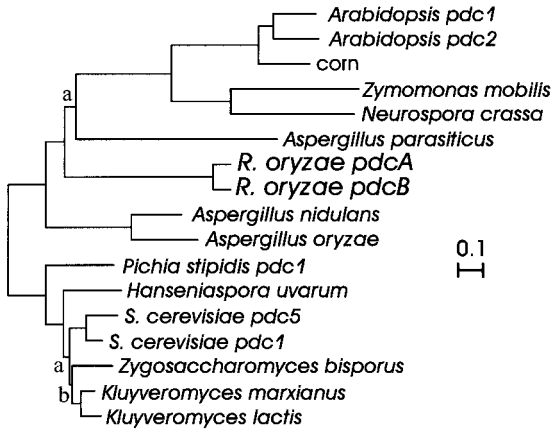

Fig. 1. Relationship of PDC subunits from numerous hosts. A most parsimonious tree for 17 PDC amino acid sequences is shown. Levels of amino acid substitutions are expressed as percentages (bar = 10%). Most of the nodes have levels of bootstrap support of 99–100%; the only exceptions are the nodes labeled **a** (81–89%) or **b** (51%). Data for *Arabidopsis* (Accession: AAB16855, CAB81915), corn [15], *Z. mobilis* [21], *N. crassa* [2], *A. parasiticus* [26], *A. nidulans* [18], *A. oryzae* [17], *P. stipidis* [19], *H. uvarum* [13], *S. cerevisiae pdc1* [14], *S. cerevisiae pdc5* (Accession: X15668), *Z. bisporus* [22], *K. marxianus* [12], and *K. lactis* [6] have been published previously.

shifted to anaerobic conditions by sparging with nitrogen decreased to 0% within minutes, while aerated cultures slowly increased to approximately 20% over the course of the experiment. It was confirmed that the low oxygen concentration for the aerated culture was due to respiration by adding the germicide Vesphene-IIse (Merck, St. Louis, MO) to the culture at the end of the experiment. The dissolved oxygen increased to 100% saturation within 10 min.

No *pdcA* or *pdcB* transcript was detected from the RNA isolated from glycerol-grown culture, but a single 1.8-kb band transcript was easily detected with each probe following the addition of glucose (Fig. 2). The signals are believed to be gene specific, since no signals were detected with hybridizations against the anti-sense primers unless more than 100 ng of target was present. Transcription of *pdcA* and *pdcB* was initiated within 1.5 h of adding glucose to the aerobically grown cultures (Fig. 3). It was at this point that one set of the fermentors was sparged with nitrogen until the end of the experiment, while the remainder of the cultures continued to be aerated. The accumulation of *pdc* transcript from cultures shifted to anaerobic growth differed significantly from those with continued aeration. After 1.5 h of nitrogen sparging (3 h after addition of glucose), the concentration of *pdcA* transcript more than doubled and pdcB transcript accumulation increased by approximately 50%. Levels of both transcripts for the anoxic cultures were almost 10-fold higher than the aerobic cultures at this time.

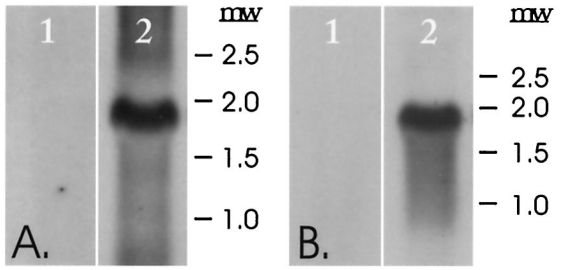

Fig. 2. Typical Northern analysis of *pdc* transcript accumulation for RNA isolated from *R. oryzae* prior to induction (lane 1) and 3 h following the addition of glucose (lane 2). RNA was hybridized with gene-specific probes that anneal to either *pdcA* (panel A) or *pdcB* (panel B). The location of molecular weight standards (in kilobases) are indicated on the right.

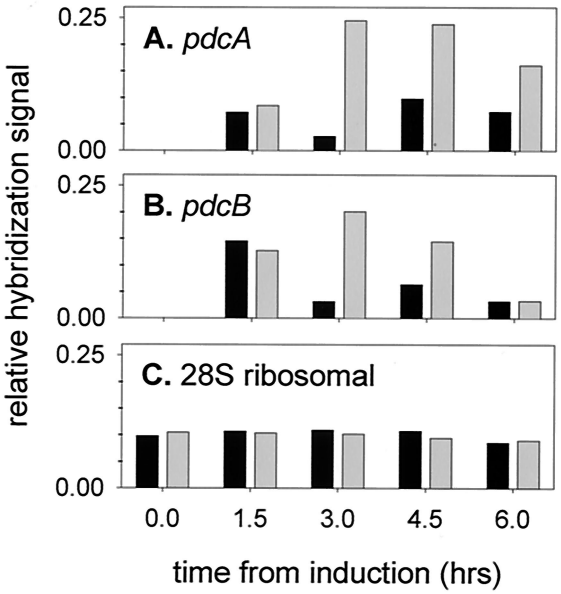

Fig. 3. Transcript accumulation during fermentative growth. *R. oryzae* cultures pre-grown in glycerol-peptone medium were induced for fermentative growth by the addition of glucose at time = 0. One set of the cultures (grey bars) was then sparged with nitrogen at 1.5 h after the addition of glucose, while the other set (black bars) continued to be sparged with air. Hybridization signals are expressed as a relative percentage of the total during the 6 h course of the experiment.

Levels of *pdc* transcripts then decreased in the anoxic culture over the next 3 h, although *pdcB* declined more rapidly than *pdcA.* Cultures with continued aeration consistently had less *pdc* transcript accumulation compared to anoxic cultures. The aerated cultures also exhibited a significant decrease, more than 60%, in accumulated *pdc* transcript at 3 h following the addition of glucose. These levels did rebound by 4.5 h, although *pdcB* increased to only approximately 50% of levels at 1.5 h after the addition of glucose.

PDC enzymatic activity generally followed the same

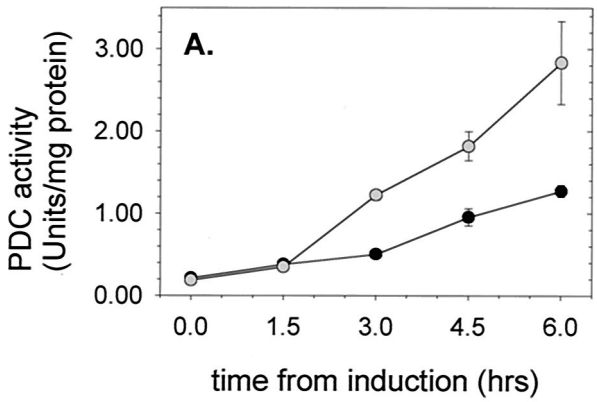

Fig. 4. PDC activity during fermentative growth. Cultures described in the previous figure were analyzed for PDC activities. One set of the cultures (grey) was sparged with nitrogen at 1.5 h after the addition of glucose, while the other set (black) continued to be sparged with air. Error bars were calculated from three independent measurements.

Table 1. Accumulation of fermentation products

Time (h) following induction[b]	Ethanol[a] (mmol/L)	
	Aerobic growth	Nitrogen sparged at 1.5 h
0	n.d.[c]	n.d.
1.5	n.d.	n.d.
3.0	n.d.	16.7 (0.9)
4.5	2.8 (0.3)	42.1 (2.5)
6.0	11.9 (0.3)	61.0 (4.3)

[a] Average of three independent measurements with std. dev. in parentheses.
[b] Transfer of mycelium to glucose-containing medium.
[c] n.d., not detected; limit of detection 2 mmol ethanol/L.

trends as transcript accumulation (Fig. 4A), with higher activities present in the anaerobically stressed culture. However, the substantial drop in *pdc* transcript levels at 3 h following glucose induction did not result in any apparent drop in Pdc activity for the aerobically grown cultures. Ethanol was first detected 3 h after adding glucose to the anaerobically shifted cultures and not until 4.5 h following the addition of glucose to the aerobic cultures (Table 1). Cultures sparged with nitrogen produced sixfold more ethanol compared to those with continued aeration.

Discussion

This work describes the first isolation of *pdc* genes from a Zygomycete fungus and shows that glucose induction is required for the initiation of transcription. Within 1.5 h of adding glucose to the glycerol grown culture, there was de novo synthesis of the *pdcA* and *pdcB* transcripts,

with enzymatic activity typically paralleling transcript accumulation. Both *pdc* genes have two to three transcriptional start sites and polyadenylation regions, which is a common feature in filamentous fungi. Oxygen availability also has a significant influence in regulating the level of *pdc* expression. There was increased accumulation of transcript, increased enzymatic activities and increased ethanol observed with nitrogen sparging, compared to cultures with continued aeration. It should be recognized that the *pdc* expression in the aerated cultures could also have been influenced by hypoxic condition due to insufficient aeration. Furthermore, the interior regions of the mycelial pellets would likely have reduced oxygen availability, regardless of the level of aeration.

It was surprising to find a precipitous drop in *pdcA* and *pdcB* transcript accumulation in the aerobic culture 3 h after glucose induction. It is speculated that this decrease in *pdc* transcript level may be associated with cAMP regulation. It is common for fungi to exhibit a transient increase in cAMP when transferred from a nonfermentable carbon source to a glucose-containing medium [7]. Such an increase could enhance the initial transcription of *pdc,* but transcription would be expected to diminish as cAMP levels stabilize. However, I was unable to identify any cAMP regulatory elements (CRE) in the upstream region of *pdcA* or *pdcB.* In the last 1.5 h of the experiment, the steady state levels of *pdcA* and *pdcB* transcripts decreased for the nitrogen sparged culture, while the PDC activity increased. The decrease of transcript may represent an inability for this obligate aerobe to sustain transcription with nitrogen sparging or might suggest that post-transcriptional regulation is involved in regulation, similar to fungal alcohol dehydrogenase genes [16].

PDC are widely distributed in fungi and plants, but are infrequently found in bacteria or animals [8]. It is common for eukaryotes to have multiple *pdc* genes, although there are only a few examples where regulation and function are well understood. Most research has focused on ethanol production in plants and yeasts, and there is still a void in the study of *pdc* genes from bacteria and filamentous fungi making it difficult to interpret the unusual grouping of these two families in the phylogenetic tree. The role of these enzymes in fermentative yeast is straightforward, while much debate continues on the necessity of ethanolic fermentation for obligate aerobes such as *Rhizopus.* There is growing evidence that alcoholic fermentation may be required for short term anaerobic survival of these aerobes [16, 18, 23]. For example, *A. nidulans* isolates were unable to survive long periods of anaerobic stress if the *adhC* gene was deleted. This is not surprising since *A. nidulans* does not appear to possess any other pathways for regenerating NAD^+ in the absence of oxygen. However, *Rhizopus* and several plants often rely on both ethanol and lactic acid as a terminal electron acceptor. Earlier work with maize found that anaerobiosis is first accompanied by a brief production of lactic acid followed by a shift to ethanol formation. It is hypothesized that this regulation occurs because anoxic conditions lead to a reduced availability of ATP, thereby decreasing export of lactic acid. Accumulation of acid lowers cytosolic pH and inhibits LDH enzyme. PDC, which typically has a lower optimal pH, is stimulated and ethanol synthesis predominates [5, 24, 25]. A similar cascade may be involved in regulation of ethanol and lactic acid in *Rhizopus,* but I have not found any evidence of lactic acid production preceding the formation of ethanol with anaerobic conditions. To more fully understand the regulation of the *R. oryzae pdc* genes, it will be necessary to perform gene deletions and re-introduction of modified *pdc* genes. However, improved techniques for genetic manipulation of this fungus will be necessary before such experiments can be performed.

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